

G protein-independent neuromodulatory action of adenosine on metabotropic glutamate signalling in mouse cerebellar Purkinje cells

Toshihide Tabata¹, Daisuke Kawakami¹, Kouichi Hashimoto¹, Hidetoshi Kassai², Takayuki Yoshida¹, Yuki Hashimotodani³, Bertil B. Fredholm⁴, Yuko Sekino⁵, Atsu Aiba² and Masanobu Kano¹

¹Department of Cellular Neuroscience, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

²Division of Cell Biology, Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0017, Japan

³Division of Neurophysiology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

⁴Department of Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden

⁵Department of Neurobiology and Behaviour, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Adenosine receptors (ARs) are G protein-coupled receptors (GPCRs) mediating the neuromodulatory actions of adenosine that influence emotional, cognitive, motor, and other functions in the central nervous system (CNS). Previous studies show complex formation between ARs and metabotropic glutamate receptors (mGluRs) in heterologous systems and close colocalization of ARs and mGluRs in several central neurons. Here we explored the possibility of intimate functional interplay between $G_{i/o}$ protein-coupled A_1 -subtype AR (A1R) and type-1 mGluR (mGluR1) naturally occurring in cerebellar Purkinje cells. Using a perforated-patch voltage-clamp technique, we found that both synthetic and endogenous agonists for A1R induced continuous depression of a mGluR1-coupled inward current. A1R agonists also depressed mGluR1-coupled intracellular Ca^{2+} mobilization monitored by fluorometry. A1R indeed mediated this depression because genetic depletion of A1R abolished it. Surprisingly, A1R agonist-induced depression persisted after blockade of $G_{i/o}$ protein. The depression appeared to involve neither the cAMP-protein kinase A cascade downstream of the alpha subunits of $G_{i/o}$ and G_s proteins, nor cytoplasmic Ca^{2+} that is suggested to be regulated by the beta-gamma subunit complex of $G_{i/o}$ protein. Moreover, A1R did not appear to affect G_q protein which mediates the mGluR1-coupled responses. These findings suggest that A1R modulates mGluR1 signalling without the aid of the major G proteins. In this respect, the A1R-mediated depression of mGluR1 signalling shown here is clearly distinguished from the A1R-mediated neuronal responses described so far. These findings demonstrate a novel neuromodulatory action of adenosine in central neurons.

(Resubmitted 5 February 2007; accepted after revision 16 March 2007; first published online 22 March 2007)

Corresponding author M. Kano: Department of Cellular Neuroscience, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan. Email: mkano@cns.med.osaka-u.ac.jp

Adenosine is a ubiquitous neuromodulator in the mammalian CNS. Adenosine is derived from neurons and glia, and accumulates in the extracellular fluid (Fredholm *et al.* 2001; Ribeiro *et al.* 2003). Adenosine activates widely distributed G protein-coupled receptors (GPCRs) named A_1 , A_{2A} , A_{2B} , and A_3 receptors (A1R, A2AR, A2B, and A3R, respectively; collectively, ARs). ARs regulate arousal level and motor activity, prevent anxiety-related behaviour and epileptiform discharges, and affect neurodegeneration,

cognition, and learning (Rudolphi *et al.* 1992; Schubert *et al.* 1997; Nyce, 1999; Fredholm *et al.* 2000; Haas & Selbach, 2000; Dunwiddie & Masino, 2001; Johansson *et al.* 2001; Ribeiro *et al.* 2003).

Inhibition of excitatory synaptic transmission is thought to be a key process for these effects of adenosine (Dunwiddie & Masino, 2001). The best studied are the inhibition of synaptic glutamate release by A1R and the inhibition of *N*-methyl-D-aspartate receptor conductance by A2AR (Haas & Selbach, 2000; Wirkner *et al.* 2000; Dunwiddie & Masino, 2001). Both types of inhibition attenuate ionotropic glutamate receptor signalling in the postsynaptic neurons. It remains unclear whether and how

K. Hashimoto and H. Kassai contributed equally to this work. This paper has online supplemental material.

adenosine in addition influences metabotropic glutamate receptor (mGluR) signalling, which plays important roles in induction of slow excitatory postsynaptic potentials (EPSPs) (Batchlor & Garthwaite, 1997; Tempia *et al.* 2001), intracellular Ca^{2+} mobilization (Llano *et al.* 1991; Finch & Augustine, 1998; Takechi *et al.* 1998), synaptic plasticity (Aiba *et al.* 1994; Conquet *et al.* 1994; Shigemoto *et al.* 1994; Ichise *et al.* 2000), production of endocannabinoids (Maejima *et al.* 2001; Maejima *et al.* 2005), and developmental synapse elimination (Kano *et al.* 1997; Ichise *et al.* 2000). Some studies have revealed that A1R and A2AR can form complexes with group-I mGluRs in non-neuronal heterologous expression systems and that immunoreactivities for A1R and mGluR1 overlap closely in several central neurons including cerebellar Purkinje cells (Ciruela *et al.* 2001; Ferre *et al.* 2002). These observations suggest the possibility of intimate functional interplay between neuronal ARs and mGluRs.

In this study, we explored possible functional interplay from native A1R to native mGluR1 in cerebellar Purkinje cells (Houamed *et al.* 1991; Masu *et al.* 1991; Reppert *et al.* 1991; Svenningsson *et al.* 1997; Ciruela *et al.* 2001). In cerebellar slice preparations, it was difficult to distinguish AR–mGluR1 interplay in Purkinje cells from synaptic modulation mediated by presynaptic ARs (Dittman & Regeher, 1996; our unpublished data). Therefore, we used isolated Purkinje cell preparations. We monitored mGluR1 signalling, using two types of G_q protein-mediated responses: an inward cation current through transient receptor potential C1 subunit-containing channels (Kim *et al.* 2003; Hartmann *et al.* 2004) and Ca^{2+} release from inositol trisphosphate receptor (IP_3R)-equipped intracellular stores (Llano *et al.* 1991; Finch & Augustine, 1998; Takechi *et al.* 1998; Miyata *et al.* 2000). We have found that A1R agonists depress both types of mGluR1-coupled responses. We have confirmed using A1R-knockout (A1R-KO) mice (Johansson *et al.* 2001) that A1R indeed mediates this depression. Surprisingly, the depression does not require $G_{i/o}$ proteins, unlike the classical A1R-mediated neuronal responses. These findings demonstrate a novel neuromodulatory action of adenosine in central neurons.

Methods

Cell culture

Cerebellar Purkinje cells from wild-type C57BL/6 mice were cultured as described elsewhere (Tabata *et al.* 2000). Briefly, perinatal embryos were caesarean-sectioned from pregnant mice deeply anaesthetized and killed with diethylether or isoflurane. The embryos were deeply anaesthetized by cooling in chilled phosphate-buffered saline and then killed by decapitation. The cerebella from these embryos were dissociated with trypsin and plated

onto plastic dishes (diameter 35 mm; Falcon 3001, Becton Dickinson, Franklin Lakes, NJ, USA) or low-fluorescence plastic films (Sumilon MS-92132, Sumitomo, Tokyo, Japan), and maintained for 11 days to 3 weeks in a medium based on 1:1 mixture of Dulbecco's modified Eagle medium and F-12 nutrients (DMF; Gibco 12400, Life Technologies, Grand Island, NY, USA). In some experiments, cerebellar neurons were dissected from newborn pups generated by mating the homozygous A1R-KO (A1R(–/–)) mice (Johansson *et al.* 2001) that were backcrossed to C57BL/6 strain according to Jackson Laboratories' specified congenic procedure. Purkinje cells were identified by their large somata and thick primary dendrites (Tabata *et al.* 2002). The experiments in this study are approved by the committees on animal experiments of Kanazawa University and Osaka University, and the procedures mentioned above fully conform to the guidelines administered by these committees.

Electrophysiology

Somatic whole-cell recordings were made from Purkinje cells cultured on the dish in a perforated-patch (Horn & Marty, 1988) or ruptured-patch mode (Marty & Neher, 1995). The pipette solution used in perforated-patch recordings consisted of (mM): 95 Cs_2SO_4 , 15 CsCl , 0.4 CsOH , 8 MgCl_2 , 10 Hepes, and $200\text{ }\mu\text{g ml}^{-1}$ amphotericin B (pH 7.35). The pipette solution used in a ruptured-patch recordings consisted of (mM): 130 K-D-glucuronate , 10 NaCl , 10 Hepes, 0.5 ethylene glycol-bis(β -aminoethylether) N,N,N',N' -tetraacetic acid, 4 Mg-ATP , 0.4 $\text{Na}_2\text{-GTP}$; the total Mg^{2+} level was adjusted to 5.2 mM with MgCl_2 ; the total K^+ level and pH were adjusted to 150.6 mM and 7.3, respectively, with KCl , KOH , and/or D-gluconic acid . The recording chamber (culture dish) was perfused at a rate of 2 ml min^{-1} ($0.7\text{--}1\text{ ml min}^{-1}$ for experiments in Fig. 8) with a saline solution. The standard saline consisted of (mM): 116 NaCl , 5.4 KCl , 1.1 NaH_2PO_4 , 23.8 NaHCO_3 , 2.3 MgCl_2 , 5.5 D-glucose , and 5 Hepes (pH 7.3, 25°C). Voltage-gated Na^+ channels and ionotropic receptors for glutamate and GABA were blocked by supplementing the saline with the following antagonists unless otherwise stated (μM): 0.3 tetrodotoxin, 10 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide, 50 $\text{D}(-)\text{-2-amino-5-phosphonopentanoic acid}$ or 10 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, and 10 $(-)\text{-bicuculline methochloride}$. Command potentials were corrected for a liquid junction potential between the pipette and bath solutions. Signals were low-pass-filtered at 0.05–2 kHz and sampled at 0.1–10 kHz, using a voltage-clamp amplifier (EPC 9/2, HEKA, Lambrecht, Germany) controlled by PULSE software (version 8.53, HEKA). We

measured mGluR1-coupled and ionotropic glutamate receptor-mediated currents and the direct effect of an A1R agonist in the perforated-patch mode (series resistance (R_{series}) $\leq 50 \text{ M}\Omega$) and $G_{\text{i/o}}$ protein-coupled inwardly rectifying K^+ currents in the ruptured-patch mode (R_{series} , $\sim 15 \text{ M}\Omega$; electronic compensation, 60%). For depicting the ramp I - V relation of the inwardly rectifying currents, the membrane potential was swept from -31 mV to -131 mV for 1 s in the saline supplemented with 10.6 mM KCl; the E_{K} estimated from the Nernst equation was -57.6 mV . We recorded the hyperpolarization-activated mixed cation current (I_{h}) in the ruptured-patch mode (R_{series} , $\sim 15 \text{ M}\Omega$; electronic compensation, 60%). In this experiment, MgCl_2 in the perfusate was replaced partially with 1 mM BaCl_2 and 1 mM CaCl_2 to minimize inwardly rectifying K^+ currents (Tabata *et al.* 2005).

Fluorometry

Purkinje cells cultured on the film were loaded with fura-2, a Ca^{2+} indicator by incubation with fura-2 acetoxymethyl ester ($5 \text{ }\mu\text{M}$, 37°C , 15 min). Then, the film with the cells was placed on a glass-based recording chamber and perfused at a rate of 1 – 1.5 ml min^{-1} with a saline solution (see above); 2.3 mM MgCl_2 was partially replaced with 2 mM CaCl_2 . $[\text{Ca}^{2+}]_{\text{i}}$ -dependent fluorescence signals were captured at 2–5 Hz, using an imaging system (Polychrome II, TILL, Planegg, Germany) attached to an inverted microscope (NA = 0.75; IX70, Olympus, Tokyo, Japan). A change in the $[\text{Ca}^{2+}]_{\text{i}}$ was expressed as a change in the ratio of somatic fluorescence signals excited at 340 and 380 nm (exposure duration, 40 and 20 ms, respectively) (change in F_{340}/F_{380}).

Drug application

Test drugs were dissolved into water to a concentration 200–1000 times higher than the final level (stock solution) and kept at -20°C or 4°C until use. The stock solutions of (RS)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and KT5720 were prepared with 10 mM Hepes buffer (pH adjusted to 7.1 with NaOH) and DMSO, respectively, and kept at -20°C until use. Adenosine was dissolved directly into the saline within 1 h before use.

Local application of agonists for mGluR1 and A1R, AMPA, and KT5720 was done by delivering saline containing each agent through a single-barrel glass pipette (i.d., 0.86 mm ; o.d., 1.5 mm) or one of the two barrels of a theta glass pipette (outer diameter, 1.5 mm ; BT150-10, Sutter, Novato, CA, USA). The diameter of a barrel opening was 150 – $200 \text{ }\mu\text{m}$. The pipette tip was located so that direct fluid stream encompassed the dendrites and soma of the examined cell (distance from the tip to the cell, 1 – 1.2 mm). Fluid flow was driven by gravity (gap between

the heights of the reservoir and the pipette tip, 20 – 40 cm). The timing of a short-term local application was regulated by an electromagnetic valve (VM8, ALA, Westbury, NY, USA) controlled by the imaging system or Master-8 (AMPI, Jerusalem, Israel). For bath application, the recording chamber was perfused with saline containing AR agonists (2 ml min^{-1}), baclofen (2 ml min^{-1}), or NF023 (1 ml min^{-1}).

In some experiments, Purkinje cells were pretreated with pertussis toxin (PTX) by adding DFM containing $50 \text{ }\mu\text{g ml}^{-1}$ PTX with or without 1% v/v bovine serum albumin to the culture medium at 1 : 100.

Data analysis

To measure the peak amplitudes of mGluR1-coupled and AMPA-evoked currents, the current records were further filtered at 1 Hz and 30 Hz off line, respectively. The data of mGluR1-coupled $[\text{Ca}^{2+}]_{\text{i}}$ rises were discarded when there was a drastic change in the kinetics between the basal and test records. The peak amplitude was measured as a difference from the prestimulus level to the maximal deflection throughout a 10 s agonist application (for both mGluR1-coupled responses) or throughout the record (for the AMPA-evoked current). The resting $[\text{Ca}^{2+}]_{\text{i}}$ is presented by the mean level over 4 s prior to agonist application. The steady-state amplitude of the time-dependent component of I_{h} was measured as a difference from the level upon a test potential-evoked capacitive transient to the level at the end of the test potential step. A numerical data group is presented as mean \pm s.e.m. throughout the text and figures. Differences between raw-value data groups were examined by ANOVA and unpaired t test unless otherwise stated. Differences between percentage-scored data groups were examined by rank score tests because the original distribution of the values could be distorted after scoring. When both percentage-scored data groups to be compared were judged to have normal distribution ($P > 0.05$, Shapiro–Wilk W test), the Van der Waerden test was used; otherwise, the Wilcoxon (Mann–Whitney U) test was used.

Results

A1R activation depresses mGluR1 signalling

First, we explored the effects of AR subtype-specific agonists on mGluR1 signalling in cultured cerebellar Purkinje cells. To ensure activation of a majority of the target receptors, we applied the corresponding agonist at a dose at least 10 times higher than the reported binding affinity (Abbracchio *et al.* 1997; Poulsen & Quinn, 1998; Klotz, 2000; Fredholm *et al.* 2001). We

evaluated the intensity of mGluR1 signalling by the peak amplitude of a *R,S*-3,5-dihydroxyphenylglycine (DHPG, a group-I mGluR-selective agonist, $50\ \mu\text{M}$)-evoked inward cation current that is coupled to mGluR1 exclusively via G_q protein (Hartmann *et al.* 2004) (Figs 1–7 and 10). We have previously reported that extracellular Ca^{2+} increases the ligand sensitivity of mGluR1 via GABA_BR (Tabata *et al.* 2002; Tabata *et al.* 2004). To exclude this effect of extracellular Ca^{2+} , we used Ca^{2+} -free saline unless otherwise stated. The peak latency of the inward current ranged typically between 2 s and 8 s (Figs 1–7 and 10). This deviation was not due to a change in drug application speed because a current evoked by AMPA delivered through the same local applicator (see below, cf. Figure 9A) did not show such a large deviation in activation kinetics (10–90% rise time, $137.2 \pm 15.0\ \text{ms}$, $n = 5$). Despite the intrinsic deviation in the kinetics, the peak amplitude of the DHPG-evoked inward current is a reliable index of mGluR1 activity because there is only a small cell-to-cell deviation in the DHPG dose dependence of the peak amplitude (Tabata *et al.* 2002; Tabata *et al.* 2004).

Bath application of normal saline did not change the peak amplitude of the inward current ($n = 7$ –8, Fig. 1A). By contrast, bath application of [R]- N^6 -(1-methyl-2-phenylethyl)adenosine (R-PIA, $50\ \text{nM}$, $n = 7$, Fig. 1B) or 2-chloro- N^6 -cyclopentyladenosine (CCPA, $500\ \text{nM}$, $n = 7$, Fig. 1C), an A1R-selective agonist,

depressed the inward current. For both agonists, the depression became evident upon application onset and continued to develop gradually for 12 min without inactivation (desensitization). The effect of R-PIA reached the plateau at 12 min of application onset as an additional 3 min application did not further reduce the peak amplitude (44.1 ± 18.0 versus $42.3 \pm 13.7\%$ of the basal level, $n = 3$, $P > 0.05$, rank score test, data not illustrated). The peak amplitude of the inward current at 12 min after the application onset of R-PIA ($41.6 \pm 9.3\%$, $n = 7$) or CCPA ($63.3 \pm 10.6\%$, $n = 7$) was significantly smaller than that of the control ($94.3 \pm 9.3\%$, $n = 8$) (Fig. 1D). The peak amplitude recovered partially after agonist offset (Fig. 1B and C).

CGS21680 ($150\ \text{nM}$, $n = 7$), an A2AR-selective agonist and 2-(1-hexynyl)-*N*-methyladenosine (HEMADO, $30\ \text{nM}$, $n = 7$), an A3R-selective agonist did not clearly depress the inward current (Figs 2A and B). The minimal peak amplitudes measured with CGS21680 ($89.5 \pm 8.3\%$ of the basal level, at 3 min after application onset) and HEMADO ($85.7 \pm 5.6\%$) were not smaller than that of the control ($94.5 \pm 8.3\%$, $n = 8$) (Fig. 2C). The results in Figs 1 and 2 together demonstrate that activation of A1R but not A2AR or A3R leads to the depression of mGluR1 signalling in Purkinje cells.

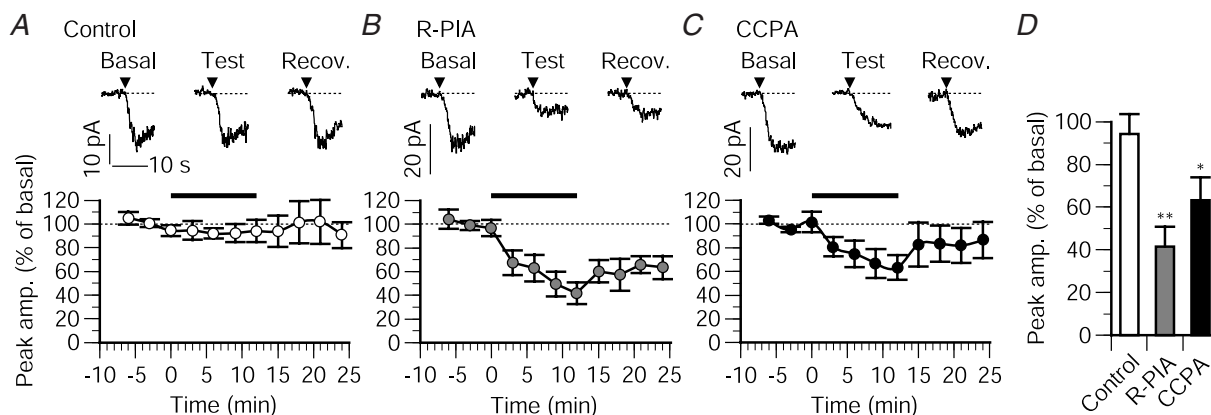


Figure 1. A1R activation depresses mGluR1 signalling

A–C, bath application of A1R-selective agonists (R-PIA, $50\ \text{nM}$, 7 cells; CCPA, $500\ \text{nM}$, 7 cells) but not the normal saline (control, 7–8 cells) depresses mGluR1-coupled inward currents evoked by DHPG ($50\ \mu\text{M}$, applied locally for 10 s) in cerebellar Purkinje cells. The cells were voltage-clamped at $-70\ \text{mV}$ in a perforated-patch whole-cell mode. Traces in each panel indicate sample responses recorded from a single cell before (Basal), during (Test), and after (Recov.) bath application. Arrowhead, DHPG onset. Each plot summarizes the mean peak amplitude of the inward currents as a function of time. For each cell, the peak amplitude is percentage scored, taking the average of three basal records as 100%. Thick bar, bath application period. The same conventions of data acquisition and presentation apply to Figs 1–6 and 10. In some cases with R-PIA, bath application of this agent was extended to seek the plateau of its effect (see Results); the commencement of the recovery time courses was aligned to a time of 12 min in the plot. D, comparison of the effects of the labelled test agents by the mean peak amplitudes at 12 min of bath application onset. Data were collected from the same cells used in A–C. $*P < 0.05$ and $**P < 0.01$ compared with the control, respectively (rank score test).

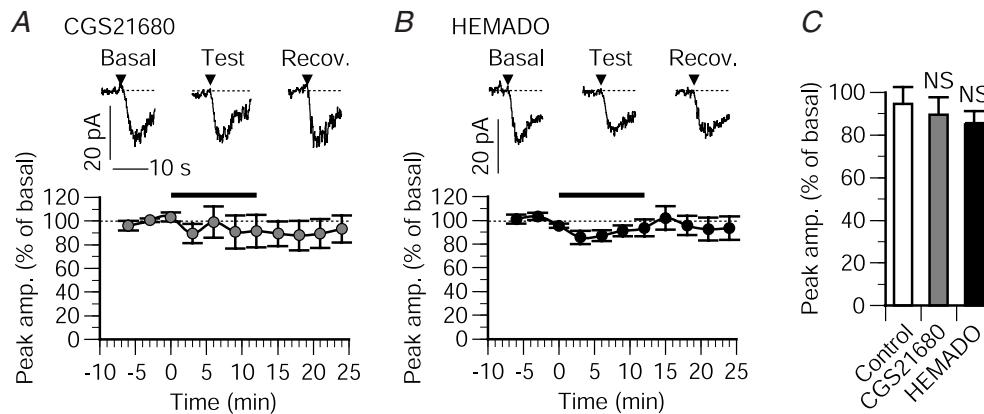


Figure 2. A2AR and A3R are not important for mGluR1 signalling depression

A and B, neither an A2AR agonist (CGS21680, bath-applied at 150 nM, 7 cells) nor an A3R agonist (HEMADO, bath-applied at 30 nM, 7 cells) depresses DHPG (50 μ M, 10 s)-evoked inward currents. C, comparison of the effects of the labelled test agents by the mean peak amplitudes at 3 min after bath application onset. Control, the normal saline (8 cells, reproduced from Fig. 1). CGS and HEMADO, data collected from the same cells used in A and B, respectively. NS, $P > 0.05$ compared with the control (rank score test).

A1R agonist-induced depression is independent of $G_{i/o}$ protein

A1R is coupled selectively to pertussis toxin (PTX)-sensitive $G_{i/o}$ protein over other G proteins (Klinger *et al.* 2002). Thus, $G_{i/o}$ protein could possibly mediate mGluR1 depression in cultured cerebellar Purkinje cells. However, this apparently conflicts with results from a previous study (Hirono *et al.* 2001), where activation of $G_{i/o}$ protein via GABA_BR augmented mGluR1 signalling in Purkinje cells in cerebellar slices.

We therefore checked whether $G_{i/o}$ protein could act on mGluR1 signalling in a different manner between culture and slice preparations. We stimulated $G_{i/o}$ protein

activation by GABA_BR with baclofen, a GABA_BR-selective agonist and measured its effect on the DHPG-evoked inward current. We used a saturating level of DHPG (500 μ M (Tabata *et al.* 2002)) in this experiment to rule out the $G_{i/o}$ protein-independent effect of baclofen which increases mGluR1's sensitivity to an unsaturating dose of DHPG (Tabata *et al.* 2004). Bath application of baclofen (1 μ M, $n = 7$) but not the normal saline ($n = 7$) significantly augmented the peak amplitude of the inward current (Fig. 3A–C). This result suggests that $G_{i/o}$ protein acts on mGluR1 signalling in the same manner in the culture and slice preparations.

A possibility therefore exists that mGluR1 signalling depression induced by A1R agonists is mediated by a

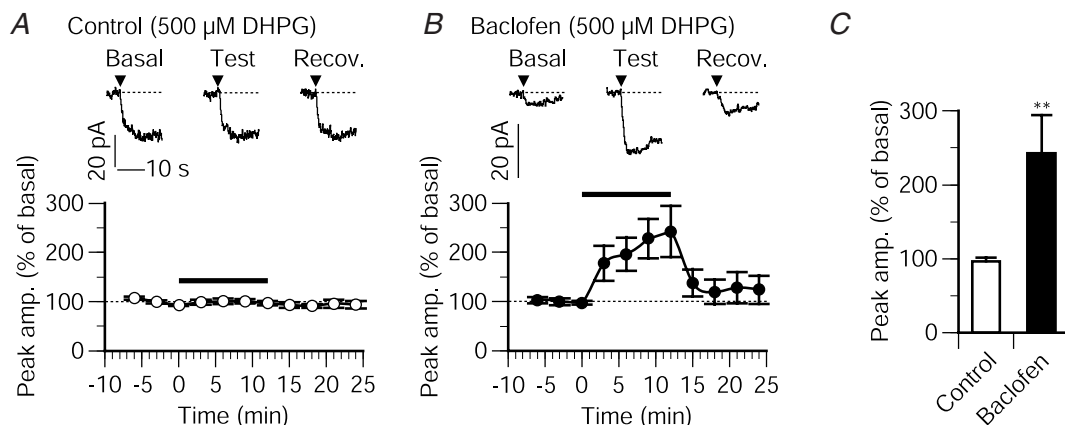


Figure 3. $G_{i/o}$ protein activation via GABA_BR augments mGluR1 signalling

A and B, bath application of a GABA_BR-selective agonist (baclofen, 1 μ M, 7 cells, B) but not the normal saline (control, 7 cells, A) induces the augmentation of DHPG (10 s)-evoked inward currents. Only in these experiments, DHPG was applied at a saturating dose (500 μ M, see Results for further explanation). C, comparison of the effects of the normal saline and baclofen by the mean peak amplitudes at 12 min after bath application onset. Data were collected from the same cells used in A and B. ** $P < 0.01$ compared with the control (rank score test).

$G_{i/o}$ protein-independent pathway. This possibility is supported by an experiment using pertussis toxin (PTX), a toxin uncoupling $G_{i/o}$ protein from G protein-coupled receptors (GPCRs). The R-PIA (50 nM)-induced depression of the inward currents persisted in Purkinje

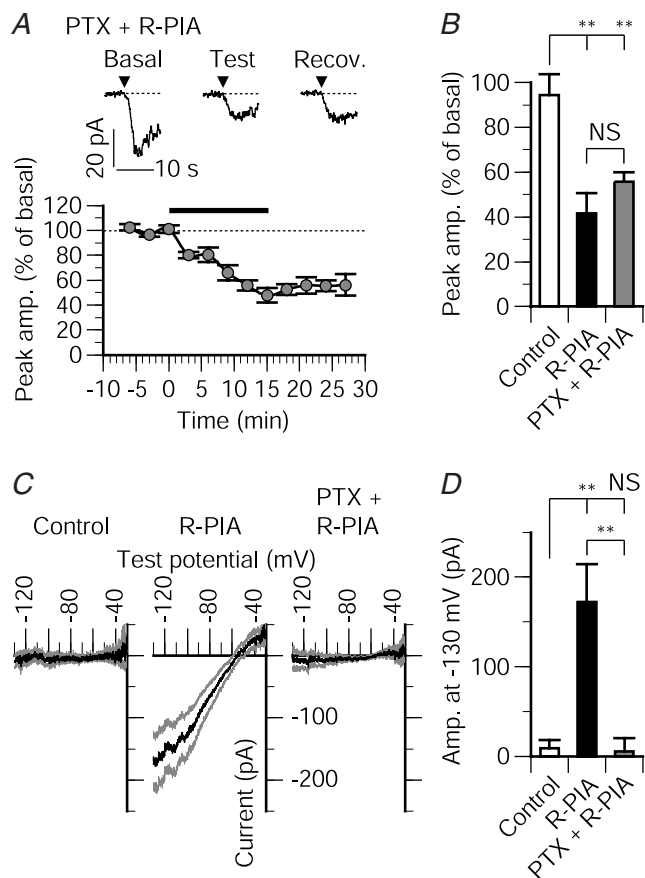


Figure 4. A1R-mediated mGluR1 signalling depression does not require $G_{i/o}$ protein

A, pretreatment with a $G_{i/o}$ protein uncoupler (PTX, 500 ng ml⁻¹, for 16 h or longer, 7 cells) does not abolish the R-PIA (50 nM, bath-applied)-induced depression of DHPG (50 μ M, 10 s)-evoked inward currents. B, comparison of the effects of the labelled test agents by the mean peak amplitudes at 12 min after bath application onset. Control, the normal saline without the PTX pretreatment (8 cells, reproduced from Fig. 1). R-PIA, R-PIA (50 nM) without the PTX pretreatment (7 cells, reproduced from Fig. 1). PTX + R-PIA, data collected from the same cells used in A. ** P < 0.01 and NS, P > 0.05, respectively (rank score test). C, R-PIA (50 nM, 9 cells) but not the normal saline (control, 11 cells) induces an inwardly rectifying current, which is suppressed by the PTX pretreatment (500 ng ml⁻¹, for 16 h or longer, 9 cells, PTX + R-PIA). Each plot indicates the mean (black line) and s.e.m. (grey lines) of the I - V relations of a current induced by the labelled agent that was extracted as a difference between the total currents evoked by voltage ramps before and after a 2 min local application of the agents. The Purkinje cells were voltage clamped in a ruptured-patch whole-cell mode. Note that the R-PIA-induced current has a reversal potential (-58.3 ± 4.9 mV) close to the E_K (-56.7 mV). D, comparison of the amplitudes at -130 mV of currents induced by the labelled test agents. Data were collected from the same cells used in C. NS, P > 0.05 and ** P < 0.01, respectively (ANOVA and unpaired t test).

cells pretreated with PTX (500 ng ml⁻¹, over 16 h) (n = 7, Fig. 4A). The peak amplitude of the inward current at 12 min after R-PIA onset ($55.8 \pm 4.1\%$ of the basal level) was significantly smaller than the control ($94.3 \pm 9.4\%$, n = 8) (Fig. 4B).

We confirmed the effectiveness of the PTX pretreatment, using a $G_{i/o}$ protein-coupled inwardly rectifying K⁺ (GIRK) current (Tabata *et al.* 2005) (Fig. 4C and D). We measured the total currents activated by a voltage ramp (from -31 mV to -131 mV) in Purkinje cells before and during application of test agents. We extracted the component induced by a test agent as a difference between these currents. The GIRK current is sensitive to Cs⁺ and activated only at membrane potentials around and negative to the equilibrium potential of K⁺ (E_K) (Tabata *et al.* 2005). To facilitate activation of the GIRK current, we used a Cs⁺-free pipette solution and raised the external K⁺ level in this experiment (see Methods). R-PIA (500 nM; n = 9; Fig. 4C and D, 'R-PIA') but not the normal saline (n = 11; Fig. 4C and D, 'Control') induced an inwardly rectifying current in PTX-untreated cells. By contrast, R-PIA did not induce any significant current in PTX-pretreated cells (500 ng ml⁻¹, over 16 h; n = 11; Fig. 4C and D, 'PTX + R-PIA'), showing that the pretreatment completely uncoupled $G_{i/o}$ protein from A1R. These results clearly demonstrate that $G_{i/o}$ protein is not required for the A1R agonist-induced depression of mGluR1 signalling.

The R-PIA-induced GIRK current (see above) shows functional coupling between A1R and $G_{i/o}$ protein in Purkinje cells. Thus, A1R could possibly mediate the augmentation of mGluR1-coupled responses like GABA_BR when A1R activates sufficient $G_{i/o}$ protein to operate its subsequent signalling cascade that modulates mGluR1 signalling (e.g. phospholipase C (PLC)-IP₃R cascade; see Discussion). We found that A1R indeed exerts such an action when strongly stimulated. In this experiment, we used 2-chloroadenosine (2-CA), an A1R-preferring agonist which is much more water soluble than other synthetic A1R agonists and thus, can be given at a high dose to achieve massive A1R stimulation in neurons (Dittman & Regeher, 1996). A high dose (30 μ M) of 2-CA augmented the inward current transiently at the early phase of drug application (n = 6, Fig. 5A). This augmentation was followed by depression that developed onward (Fig. 5A). The peak amplitude at 3 min after 2-CA onset ($168.7 \pm 30.8\%$ of the basal level; Fig. 5C) was significantly larger than the control ($94.5 \pm 8.0\%$, n = 8). The peak amplitude at 12 min after 2-CA onset ($55.8 \pm 12.5\%$, Fig. 5C, right) was significantly smaller than the control ($94.3 \pm 9.3\%$, n = 8). Pretreatment with PTX (500 ng ml⁻¹, over 16 h) abolished selectively the transient augmentation (n = 7, Fig. 5B). The peak amplitude was not augmented at 3 min after 2-CA onset ($83.0 \pm 6.3\%$; Fig. 5C, left) while it was

significantly more reduced ($69.0 \pm 3.8\%$) than the control at 12 min after 2-CA onset (Fig. 5C, right). These results indicate that the $G_{i/o}$ protein-independent depression of mGluR1 signalling masks the $G_{i/o}$ protein-dependent augmentation unless A1R is stimulated strongly. The results in Figs 3–5 together clearly demonstrate that a $G_{i/o}$ protein-independent pathway underlies the A1R agonist-induced depression of mGluR1 signalling.

mGluR1 signalling depression is independent of signalling cascades downstream of major G proteins

$G_{i/o}$ protein inhibits cAMP production by adenylyl cyclase and is thereby negatively coupled to cAMP and its subsequent signalling cascade. We examined the sensitivity of mGluR1 signalling to a change in cAMP level, using forskolin ($20 \mu\text{M}$), an adenylyl cyclase activator. The peak amplitudes of DHPG-evoked inward currents at 6 min ($107.7 \pm 9.0\%$ of the basal level, $n = 7$) and 12 min ($99.5 \pm 7.9\%$, $n = 7$) of forskolin onset were not significantly different from the control ($92.0 \pm 4.4\%$, $n = 7$ and $94.3 \pm 9.3\%$, respectively) (Fig. 6A and B). The cAMP level should indeed be increased in this experiment because the same dose of forskolin augmented the hyperpolarization-activated, mixed cation current (I_h) that is sensitive to cAMP in cerebellar neurons (Saitow & Konishi, 2000) (Fig. 6C and D). These results suggest that cAMP-dependent mechanisms cannot explain mGluR1 signalling depression.

Some studies using heterologous expression systems (Miyashita & Kubo, 2000; Tateyama & Kubo, 2006) show that a GPCR may interact with multiple classes of G proteins, depending on the cellular environment. If A1R could interact with not only $G_{i/o}$ proteins but also G_q protein, A1R could possibly affect directly signalling from mGluR1 to G_q protein. For example, a prolonged activation of A1R could consume G_q protein, and this could result in the apparent depression of mGluR1 signalling. To test the possibility of A1R– G_q protein coupling, we checked whether R-PIA evokes the G_q protein-mediated inward current (see Introduction) like DHPG (mGluR1 agonist). Local application of R-PIA (500 nM) did not produce any detectable current in the cells that showed DHPG-evoked inward currents (Fig. 7A). The maximal inward deflection during R-PIA application ($1.5 \pm 0.8 \text{ pA}$) was not different with the control ($2.0 \pm 0.7 \text{ pA}$) and was significantly smaller than that of DHPG ($24.6 \pm 3.8 \text{ pA}$) ($n = 7$, Fig. 7B). This result suggests that A1R– G_q protein coupling cannot explain mGluR1 depression.

A1R agonist-induced depression is specific for metabotropic glutamate signalling

To exclude a possibility that A1R-mediated mGluR1 signalling depression is an artefact, we checked the direct effect of R-PIA on Purkinje cells. For measuring the mGluR1-coupled inward current (Figs 1–7 and 10, except

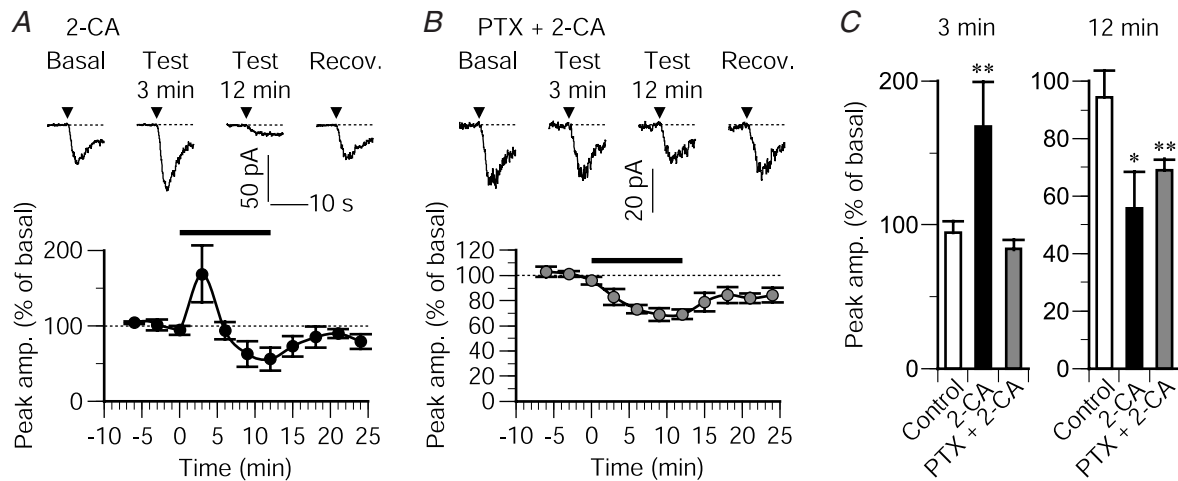


Figure 5. A1R exerts the opposite actions on mGluR1 signalling through $G_{i/o}$ protein-dependent and -independent pathways

A and B, bath application of a high dose of a potent AR agonist (2-CA, $30 \mu\text{M}$) augments DHPG ($50 \mu\text{M}$, 10 s)-evoked inward currents transiently prior to inducing depression in PTX-untreated Purkinje cells (6 cells, A) but not in PTX-pretreated cells (500 ng ml^{-1} , over 16 h, 6 cells, B). Test 3 min and Test 12 min, responses at 3 min and 12 min after 2-CA onset. C, comparison of the effects of the labelled test agents by the mean peak amplitudes at 3 min and 12 min after bath application onset. Control, the normal saline without the PTX pretreatment (8 cells, reproduced from Fig. 1). 2-CA, 2-CA without the PTX pretreatment, data collected from the cells used in A. PTX + 2-CA, 2-CA with the PTX pretreatment, data collected from the same cells used in B. * $P < 0.05$ and ** $P < 0.01$ compared with the control, respectively (rank score test).

Fig. 4C and D), we used the Cs⁺-containing pipette solution. With this solution, local application of R-PIA (50 nM) little changed the holding current level and input resistance measured by voltage steps (Fig. 8A). The relative holding current levels to the basal value (-89.1 ± 18.1 pA) at 3–4 min and 13–14 min of R-PIA onset were $104.0 \pm 4.3\%$ and $96.4 \pm 7.2\%$, respectively ($n = 5$, Fig. 8A and B). The relative input resistances to the basal value (282 ± 82 M Ω) at 3–4 min and 12–13 min R-PIA onset were $94.7 \pm 5.7\%$ and $101.6 \pm 5.1\%$, respectively (Fig. 8A and C). As well, local application of CCPA (500 nM, $n = 3$), 2-CA (30 μ M, $n = 3$), or adenosine (400 nM, $n = 4$) did not significantly change the holding current level (basal value, -106.8 ± 13.6 pA; at 3–4 min of drug onset, $96.4 \pm 5.7\%$, $101.6 \pm 3.2\%$, and $100.2 \pm 6.6\%$; at 12–13 min of drug

onset, $96.0 \pm 10.5\%$, $98.3 \pm 4.3\%$, and $103.6 \pm 11.5\%$, respectively) and input resistance measured by +5 mV voltage steps (basal, 236.5 ± 24.7 M Ω ; at 3–4 min of drug onset, $121.4 \pm 17.1\%$, $92.9 \pm 3.1\%$, and $104.2 \pm 4.8\%$; at 12–13 min of drug onset, $128.7 \pm 18.0\%$, $102.3 \pm 4.7\%$, $108.4 \pm 8.4\%$, respectively) ($P > 0.05$, unpaired t test for the raw data; Fig. 8D). Thus, the depression of the inward current is not attributable to a change in the background conductance, which could shift the actual holding potential imposed to the cell membrane and thereby could affect the amplitude of the inward current.

Bath application of R-PIA (50 nM, 3–13 min) did not change the peak amplitude of an ionotropic glutamate receptor-mediated inward current evoked by local application of AMPA (10 μ M, 100 ms) (546.3 ± 150.6 pA, changed by $0.5 \pm 5.7\%$ at 12–13 min of R-PIA onset, $n = 5$) (Fig. 9A and B). This result again suggests that A1R activation does not shift the holding potential. It also suggests that A1R agonist-induced depression occurs specifically for metabotropic glutamate signalling but not for ionotropic glutamate signalling.

Physiological levels of adenosine depress mGluR1 signalling

We examined how physiological levels of the endogenous AR agonist adenosine affect mGluR1 signalling. In some brain regions, microdialysis detects ~ 40 nM of adenosine when no drug is administered and ~ 400 nM of adenosine following pharmacological inhibition of the adenosine uptake mechanisms (Ballarin *et al.* 1991). These values may indicate the range of extracellular concentrations of adenosine under physiological conditions. Adenosine at both levels (40 nM, $n = 8$, Fig. 10A and 400 nM, $n = 7$, Fig. 10B) depressed the DHPG-evoked inward current. The depression developed

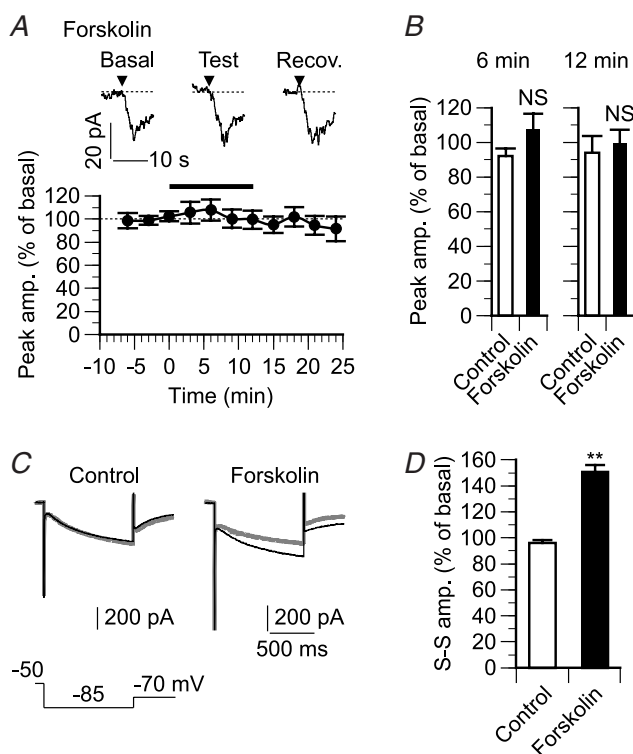


Figure 6. cAMP insensitivity of mGluR1 signalling

A, bath application of an adenylyl cyclase activator (forskolin, 20 μ M) has little effect on the amplitude of DHPG (50 μ M, 10 s)-evoked inward currents (7 cells). B, comparison of the effects of the normal saline (7–8 cells, reproduced from Fig. 1) and forskolin (7 cells, data collected from the cells used in A) by the mean peak amplitudes at 6 min and 12 min after bath application onset. NS, $P > 0.05$ (rank score test). C, forskolin (20 μ M) indeed changes cAMP level as it augments I_h . Each set of superimposed traces indicates sample responses of a cell obtained before (grey thick line) and after (thin black line) a 2 min local application of forskolin. Schematics below the traces, voltage protocol. The Purkinje cells were voltage clamped in the ruptured-patch whole-cell mode. D, comparison of the steady-state amplitudes of the time-dependent components of I_h after application of the normal saline (Control, 11 cells) or forskolin (8 cells). $**P < 0.01$ compared with the control, rank score test.

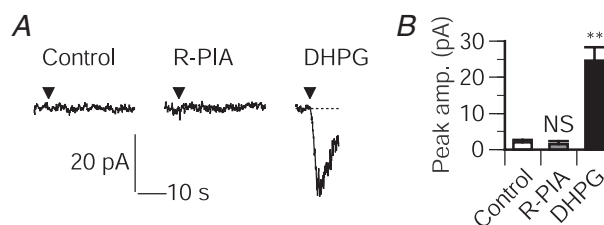


Figure 7. G_q protein is not coupled to A1R

A, local application of R-PIA (500 nM, 30 s) as well as the normal saline (30 s) does not evoke an inward current, unlike DHPG (50 μ M, 10 s). Traces indicate the sample responses of a cell. Arrowheads, local application onset. Holding potential, -70 mV (perforated-patch whole-cell mode). The GIRK current was blocked with 5 mM Cs⁺ in the saline (Tabata *et al.* 2005). B, comparison of the mean maximal inward deflections during application of the labelled test drugs. NS, $P > 0.05$ and $**P < 0.01$ compared with the control, respectively (ANOVA and paired t test).

upon adenosine onset and lasted throughout a 12 min application. When compared at 12 min after application onset, the peak amplitude of the inward current ($70.9 \pm 5.6\%$ of the basal level with 40 nM adenosine,

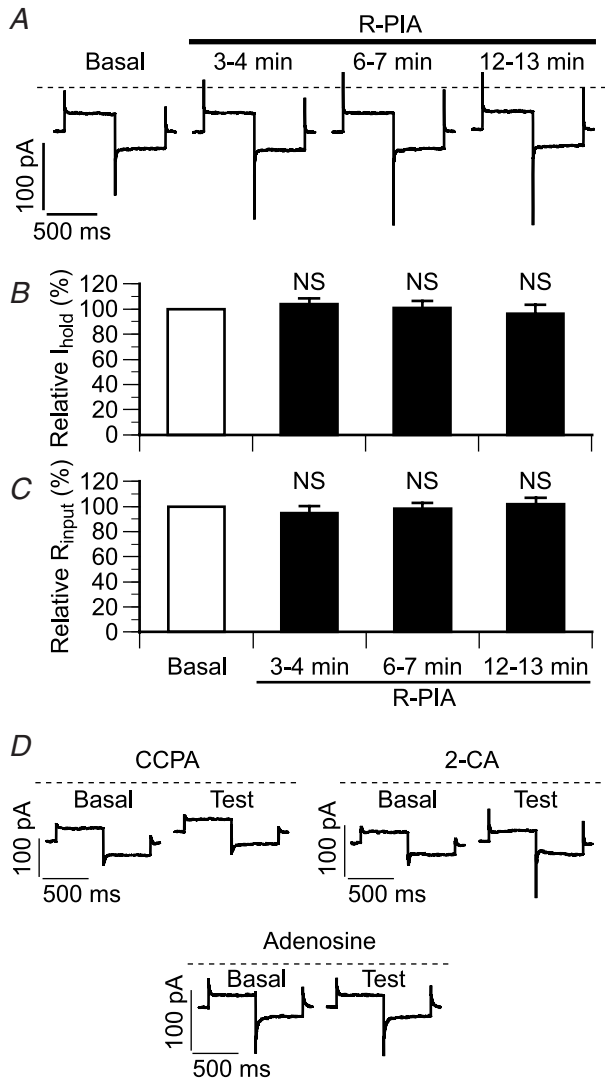


Figure 8. A1R-mediated mGluR1 depression is not associated with a change in the background conductance

A, sample voltage step-evoked currents of a Purkinje cell recorded before (Basal) and during local application of R-PIA (50 nM). Each trace indicates the average of three records obtained at the labelled time after R-PIA onset. Dotted line, zero-current level. The input resistance (R_{input}) was estimated from a difference between mean current levels over the last 95 ms of ± 5 mV steps. Holding potential, -70 mV (perforated-patch mode with the Cs^+ -containing pipette solution). B and C, the mean holding current levels (I_{hold}) (average level over a 95 ms period prior to the first voltage step; an inward deflection indicated as a positive value) and mean R_{input} at the labelled period. For each cell, the basal value was taken as 100%. $n = 5$ cells. NS, $P > 0.05$ compared with the basal values (paired t test for the raw data). D, sample voltage step (± 5 mV)-evoked currents before (Basal) and during (at 12–13 min after drug onset, Test) local application of CCPA (500 nM), 2-CA (30 μM), or adenosine (400 nM). Each pair of traces was obtained from a different cell, using the same procedures as in A.

$67.0 \pm 5.7\%$ with 400 nM adenosine) was significantly more reduced than the control ($94.3 \pm 9.3\%$, $n = 8$) (Fig. 10C). The extent of the depression did not display obvious adenosine dose dependency (see Discussion). The peak amplitude recovered almost completely to the basal level after the offset of 40 nM adenosine (Fig. 10A). These results suggest that A1R may mediate a depressant effect on mGluR1 signalling in response to adenosine in the cerebrospinal fluid under physiological conditions.

A1R activation also depresses mGluR1-coupled intracellular Ca^{2+} mobilization

Finally, we performed fluorometry to examine whether A1R activation depresses mGluR1-coupled intracellular Ca^{2+} mobilization that is mediated by downstream signalling molecules and effectors different from those of the mGluR1-coupled inward current (see Introduction).

DHPG evoked a rise in the $[\text{Ca}^{2+}]_i$ (Fig. 11). Since this response is due mostly to Ca^{2+} release from the

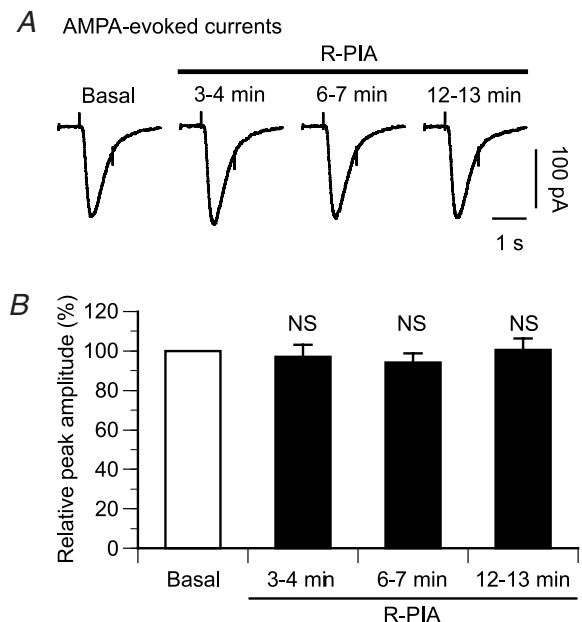


Figure 9. A1R-mediated depression is not observed for AMPAR-mediated signalling

A, sample AMPA (10 μM)-evoked currents of a Purkinje cell recorded before (Basal) and during bath application of R-PIA (50 nM). Each trace indicates the average of three records obtained at the labelled time after R-PIA onset. AMPA-containing saline was applied for 100 ms locally through one of the two barrels of a theta pipette, and then the control saline was applied for 1 s through the other barrel to wash out AMPA. Holding potential, -70 mV (perforated-patch mode with the Cs^+ -containing pipette solution and the standard saline without a blocker against AMPA-type glutamate receptors). B, the mean peak amplitudes of the AMPA-evoked currents at the labelled period. For each cell, the basal value was taken as 100%. Data obtained from 5 cells. NS, $P > 0.05$ compared with the basal values (paired t test for the raw data).

intracellular store (Sato *et al.* 2004), frequent DHPG application in a Ca^{2+} -free saline leads to store depletion. To minimize the influence of such rundown, we applied DHPG with a prolonged interval (13 min) in a Ca^{2+} -containing saline (Figs 11–13). When co-applied with DHPG ($50\text{ }\mu\text{M}$), R-PIA depressed the peak amplitude of the $[\text{Ca}^{2+}]_i$ rise to $47.0 \pm 30.8\%$ of the basal level ($n = 3$, Fig. 11A). This depression became evident within 1–4.4 s after co-application onset ($n = 3$, Fig. 11A, 'Superimposed'; note that the response to DHPG alone continues to grow for ~ 10 s, whereas the response to co-application of R-PIA and DHPG decelerates after 4.4 s of drug onset). This result suggests that the A1R agonist-induced depression of mGluR1 signalling can develop within a few seconds.

To determine the dose dependence of depression at the steady-state, we compared the DHPG ($5\text{ }\mu\text{M}$)-evoked $[\text{Ca}^{2+}]_i$ rises before and after a 12 min application of various doses of R-PIA (Fig. 11B and C). Depression was detectable at doses of 5 nM or higher, and reached the maximum at a dose of 50 nM (peak amplitude after R-PIA application, $64.5 \pm 10\%$ of the basal level, $n = 7$). The mean extent of depression decreased at higher doses ($80.4 \pm 12.9\%$, $n = 10$ at 500 nM; $77.0 \pm 6.7\%$, $n = 7$ at $5\text{ }\mu\text{M}$). A small fraction of the cells examined with 500 nM to $5\text{ }\mu\text{M}$ R-PIA (3 out of 17 cells) displayed augmentation of the DHPG-evoked $[\text{Ca}^{2+}]_i$ rise. Thus, strong activation of A1R might cause augmentation of mGluR1 signalling as well as depression, and this might apparently reduce the net extent of depression.

The R-PIA (50 nM, 12 min)-induced depression of the DHPG ($5\text{ }\mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rise was resistant to treatment with a $\text{G}_{i/o}$ protein inhibitor, PTX (500 ng ml^{-1} , over 16 h before the recording) or NF023 ($10\text{ }\mu\text{M}$; the

cell was perfused with NF023 for >15 min prior to and throughout the recording) (Fig. 12A). The peak amplitudes after R-PIA application ($50.6 \pm 7.0\%$ of the basal, $n = 8$ with PTX; $75.0 \pm 5.5\%$, $n = 9$ with NF023) were significantly smaller than that of the control ($108.7 \pm 9.1\%$, $n = 11$) and not different from that of the untreated cells ($64.5 \pm 10\%$, $n = 7$) (Fig. 12B). This result supports the $\text{G}_{i/o}$ protein-independence of A1R agonist-induced depression of mGluR1 signalling.

Moreover, local application of KT5720, a protein kinase A (PKA) inhibitor failed to mimic the R-PIA-induced depression of the DHPG ($5\text{ }\mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rise (Fig. 12C). The vehicle used for dissolving KT5720 (DMSO, 0.001% v/v, 12 min) by itself slightly depressed the peak amplitude ($81.6 \pm 12.9\%$, $n = 8$) (Fig. 12D). KT5720 ($1\text{ }\mu\text{M}$, 12 min) did not show further reduction ($88.8 \pm 12.9\%$, $n = 9$) (Fig. 12D). This result suggests the PKA independence of A1R agonist-induced depression of mGluR1 signalling.

We compared the effect of R-PIA on the DHPG ($5\text{ }\mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rise between cells derived from wild-type (WT) mice and the A1R-KO mice. R-PIA depressed the peak amplitude in WT cells ($64.5 \pm 10.0\%$, $n = 7$) but not in A1R-KO cells ($99.4 \pm 8.7\%$, $n = 7$) (Figs 13A and B). This result provides genetic evidence for the involvement of A1R in the A1R agonist-induced depression of GluR1 signalling.

A previous study (Hirono *et al.* 2001) has suggested that in cerebellar Purkinje cells, $\text{G}_{i/o}$ protein activation results in Ca^{2+} release from the intracellular stores (see Discussion), and this may underlie the modulation of mGluR1-coupled responses. However, such modulation cannot explain the R-PIA-induced depression of mGluR1 signalling studied here. The resting $[\text{Ca}^{2+}]_i$ did not much

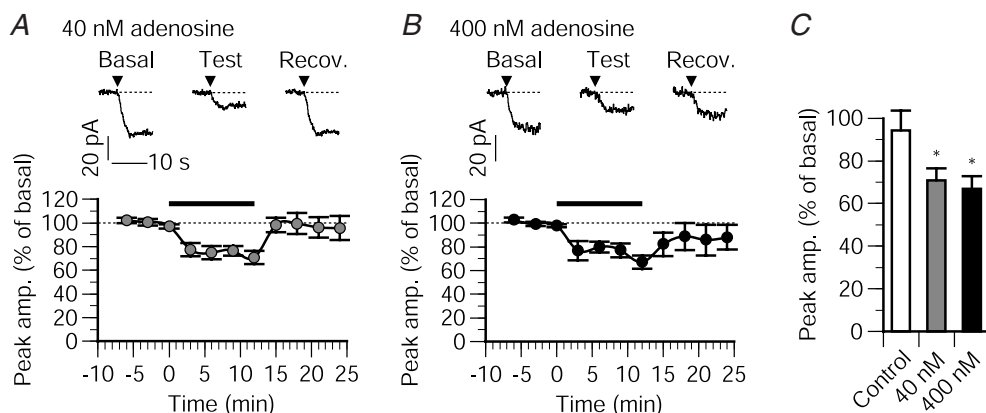


Figure 10. Physiological levels of adenosine depress mGluR1 signalling

A and B, bath application of an endogenous A1R agonist (adenosine) at physiological concentrations (40 nM, 8 cells; 400 nM, 7 cells) depresses DHPG ($50\text{ }\mu\text{M}$, 10 s)-evoked inward currents. C, comparison of the effects of the labelled test agents by the mean peak amplitudes at 12 min after bath application onset. Control, the normal saline (8 cells, reproduced from Fig. 1). 40 nM and 400 nM, data with adenosine collected from the same cells used in A and B, respectively. * $P < 0.05$ compared with the control (rank score test).

change after R-PIA treatment (50 nM, 12 min) in the WT cells ($91.8 \pm 2.8\%$ of the basal level, $n = 7$), and this extent of change was not different from that of the A1R-KO cells ($96.6 \pm 2.2\%$, $n = 10$) (Fig. 13C). This result clearly shows that with a dose of R-PIA that depresses the mGluR1-coupled inward current and $[\text{Ca}^{2+}]_i$ rise, A1R does not mediate a change in the resting $[\text{Ca}^{2+}]_i$.

Furthermore, a physiological dose (400 nM, 12 min) of adenosine depressed the peak amplitude of the DHPG ($5 \mu\text{M}$)-evoked $[\text{Ca}^{2+}]_i$ rise in WT cells ($81.2 \pm 5.6\%$ of the basal level, $n = 17$) but not in A1R-KO cells ($107.5 \pm 11.6\%$, $n = 10$) (Fig. 13D and E) without affecting the resting $[\text{Ca}^{2+}]_i$ ($102.0 \pm 1.6\%$ for the WT cells, $101.3 \pm 1.6\%$ for the A1R-KO cells, Fig. 13F). This result suggests the A1R-mediated depression of mGluR1-coupled Ca^{2+} mobilization may occur under physiological conditions.

Discussion

We found that several A1R agonists (R-PIA, Fig. 1B and D; CCPA, Fig. 1C and D; and 2-CA, Fig. 4A and B) depressed the mGluR1-coupled inward cation current in cerebellar Purkinje cells. The A2AR- and A3R-selective agonists (CGS21680 and HEMADO, respectively) failed to depress the inward currents (Fig. 2), suggesting that neither A2AR nor A3R mediates the depression. The involvement of A2BR could not be directly assessed for lack of a commercially available A2BR-selective agonist. However, A2BR does not seem to be important for the depression studied here because R-PIA and CCPA at the doses used (50 and 500 nM, respectively; Fig. 1B and C) should only minimally activate A2BR (affinities for these agonists are $4\text{--}40 \mu\text{M}$ (Poulsen & Quinn, 1998; Klotz, 2000; Fredholm *et al.* 2001)). Genetic depletion of A1R completely abolished the depression of the mGluR1-coupled $[\text{Ca}^{2+}]_i$ rise (Fig. 13). These results together unequivocally demonstrate that activation of A1R but not the other AR subtypes leads to the depression of mGluR1 signalling in Purkinje cells (we hereafter term this effect A1R–mGluR depression).

A1R has a high coupling selectivity to the $G_{i/o}$ class of G proteins (Klinger *et al.* 2002). R-PIA evoked the $G_{i/o}$ protein-coupled inwardly rectifying K^+ (GIRK) currents (Tabata *et al.* 2005) (Fig. 4C and D), showing that A1R is indeed coupled to $G_{i/o}$ proteins in Purkinje cells. Nevertheless, activation of GABA_BR , another $G_{i/o}$ protein-coupled receptor failed to mimic A1R–mGluR1 depression (Fig. 3). A1R–mGluR1 depression was resistant to $G_{i/o}$ protein inhibitors (Figs 4A and B, 5B and C and 12A and B). These results strongly suggest that A1R–mGluR1 depression does not require $G_{i/o}$ proteins.

We further assessed the involvement of signalling cascades downstream of the major G proteins. Prolonged application of forskolin had little effect on the amplitude of the inward current (Fig. 6A and B) although it indeed facilitated cAMP production by adenylyl cyclase (Fig. 6C and D). A PKA inhibitor had little effect on the amplitude

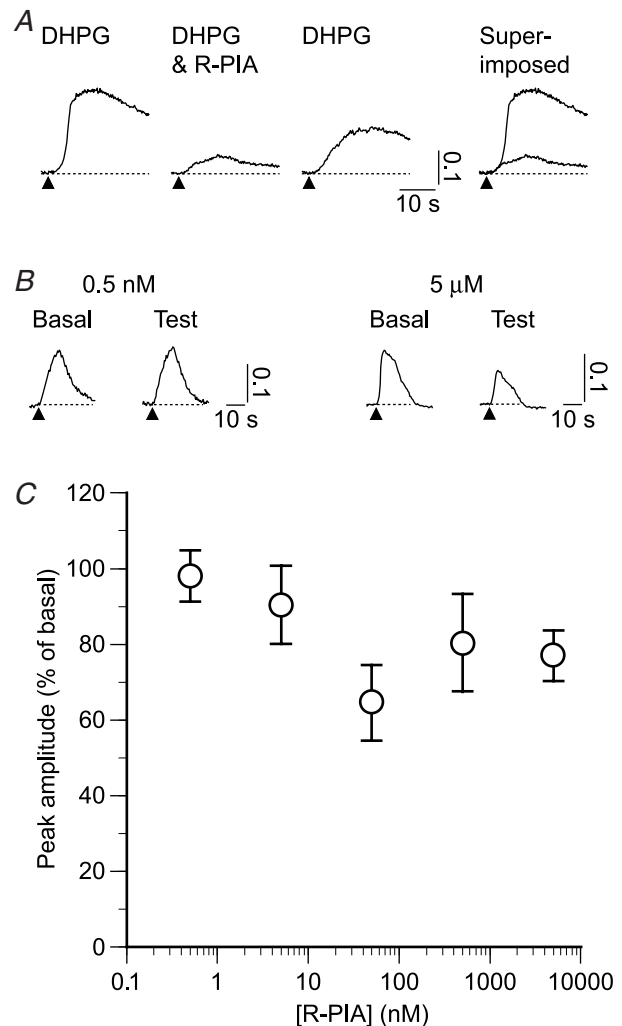


Figure 11. A1R-mediated depression is also seen for mGluR1-coupled intracellular Ca^{2+} mobilization

A, co-applied R-PIA (50 nM, & R-PIA) reduces the DHPG ($50 \mu\text{M}$)-evoked $[\text{Ca}^{2+}]_i$ rise in wild-type (WT) Purkinje cells. From left to right: sample responses of a cell to local application of the labelled agents (10 s from arrowheads) obtained consecutively at an interval of 13 min. Superimposed, the left two traces are superimposed and justified at application onset; note that the response to co-applied DHPG and R-PIA decelerates after 4.4 s of drug onset. The saline contained 2 mM Ca^{2+} . Vertical calibration bars, change in F_{340}/F_{380} . B and C, R-PIA dose dependence of depression of the DHPG ($5 \mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rise. In this and the following figures, the saline contained 2 mM Ca^{2+} . B, each set of traces indicates sample responses of a cell before (Basal) and after (Test) a 12 min local application of the labelled dose of R-PIA. C, mean peak amplitude of the $[\text{Ca}^{2+}]_i$ rise after R-PIA application plotted against logarithmic-scaled R-PIA dose. $n = 5\text{--}10$ cells for each point.

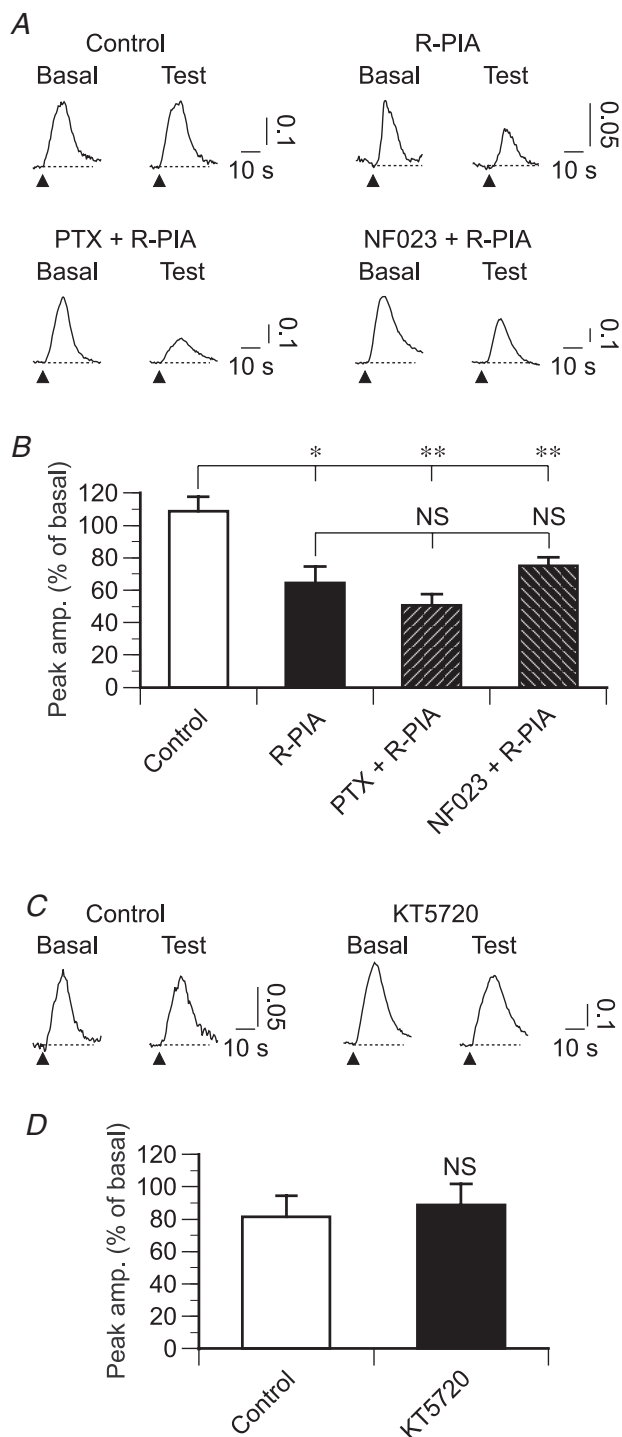


Figure 12. A1R-mediated depression of mGluR1-coupled intracellular Ca^{2+} mobilization is independent of $\text{G}_{i/o}$ protein or PKA

A and B, R-PIA-induced depression of the DHPG ($5 \mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rise persists after inhibition of $\text{G}_{i/o}$ protein. A, each set of traces indicates sample responses of a cell obtained before and after a 12 min local application of the normal saline (Control) or 50 nM R-PIA. PTX +, cell pretreated with PTX (500 ng ml^{-1}) for >16 h before the recording. NF023 +, cell treated with NF023 ($10 \mu\text{M}$) continuously for >15 min prior to and throughout the recording. B, comparison of the mean peak amplitudes of the $[\text{Ca}^{2+}]_i$ rises after application of the

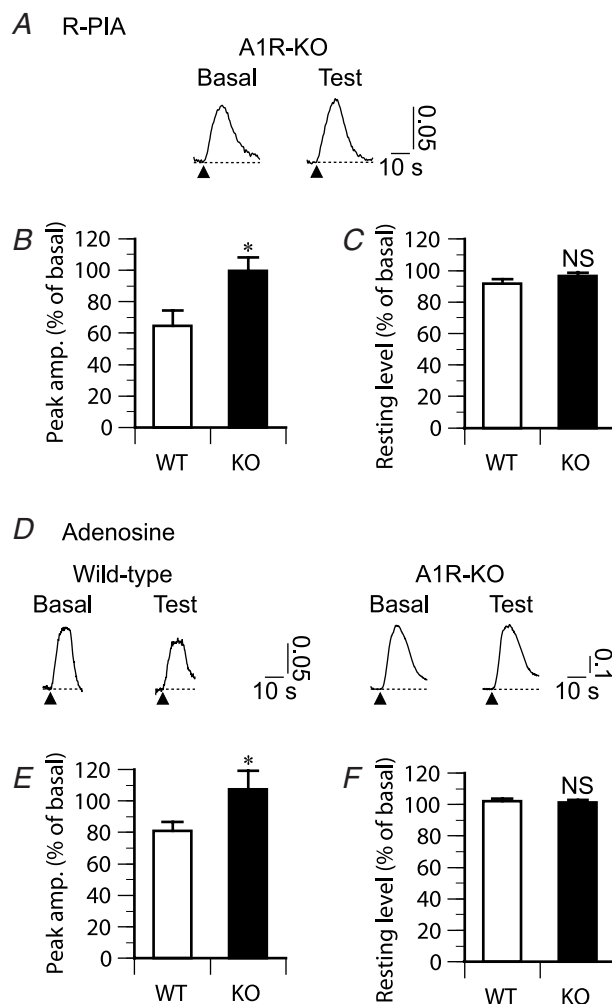


Figure 13. Genetic depletion of A1R abolishes A1R-mediated mGluR1 depression

A–F, R-PIA or adenosine depresses the DHPG-evoked $[\text{Ca}^{2+}]_i$ rise in WT Purkinje cells but not in A1R-KO cells without affecting the resting $[\text{Ca}^{2+}]_i$. A and D, each set of traces indicates sample DHPG ($5 \mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rises obtained from a cell before and after a 12 min local application of 50 nM R-PIA or 400 nM adenosine. B, C, E, and F, comparisons of the mean peak amplitudes of the $[\text{Ca}^{2+}]_i$ rises and the mean resting $[\text{Ca}^{2+}]_i$ after application of R-PIA (B and C) or adenosine (E and F). $n = 7$ WT cells (data reproduced from the previous figure) and $n = 10$ A1R-KO cells in B and C, and 17 WT cells and 10 A1R-KO cells in E and F. * $P < 0.05$ and NS, $P > 0.05$ compared with the WT cells (rank score test).

normal saline or R-PIA. $N = 7$ –11 cells for each group. * $P < 0.05$, ** $P < 0.01$, and NS, $P > 0.05$ (rank score test). C and D, inhibition of PKA does not mimic A1R-mediated depression. C, each set of traces indicates sample DHPG ($5 \mu\text{M}$, 10 s)-evoked $[\text{Ca}^{2+}]_i$ rises of a cell obtained before and after a 12 min local application of DMSO (0.01% v/v, vehicle for KT5720, Control) or $1 \mu\text{M}$ KT5720. D, comparisons of the mean peak amplitudes of the $[\text{Ca}^{2+}]_i$ rises after application of the test drugs. $n = 8$ –9 cells for each group. NS, $P > 0.05$ compared with the control (rank score test).

of the mGluR1-mediated $[Ca^{2+}]_i$ rise (Fig. 12C and D). Thus, the cAMP-PKA signalling cascade is not responsible for mGluR1 signalling depression. These results also suggest that A1R does not exert the depression via the α subunit of $G_{i/o}$ and G_s proteins that regulate adenylyl cyclase. In certain cell types, the $\beta\gamma$ subunit complex of $G_{i/o}$ protein may weakly activate PLC (for review, see (Selbie & Hill, 1998)) that is coupled to Ca^{2+} release from the IP_3 R-equipped intracellular stores. A previous study (Hirono *et al.* 2001) has suggested that in cerebellar Purkinje cells, $G_{i/o}$ protein activation results in Ca^{2+} release from the intracellular stores, and this may underlie the modulation of mGluR1-coupled responses. However, such $G_{i/o}$ protein/ Ca^{2+} -dependent modulation cannot explain A1R-mGluR1 depression, because with the dose of R-PIA used, there was no change in the resting $[Ca^{2+}]_i$ (Fig. 13C). Moreover, R-PIA did not evoke the G_q protein-mediated inward current (Hartmann *et al.* 2004) (Fig. 7). Thus, A1R should not affect directly the mGluR1- G_q protein cascade that mediates the inward current and $[Ca^{2+}]_i$ rise. These results together suggest that A1R mediates mGluR1 signalling depression independently of the major G proteins and their subsequent signalling cascades. To our knowledge, all classical A1R-coupled neuronal responses described so far are mediated by $G_{i/o}$ protein (Fredholm *et al.* 2001). A1R-mGluR1 depression may represent the first example of $G_{i/o}$ protein-independent neuronal function of native ARs in central neurons.

Under the condition used for measuring the A1R-mediated depression of the inward current (with Cs^+ -containing pipette solution), the A1R agonists did not affect the background conductance (Fig. 8) and ionotropic glutamate receptor current (Fig. 9). These results suggest that A1R-mGluR1 depression results from specific modulation of mGluR1 signalling.

A1R activation depressed both the inward current (Fig. 1) and the $[Ca^{2+}]_i$ rise (Fig. 11) which are mediated by different downstream signalling molecules and effectors (see Introduction) without the aid of the major G proteins, the primary diffusible messengers linking GPCRs to remote targets (see above). Thus, A1R-mGluR1 depression might depend on a local molecular interaction that modulates the early step of the mGluR1 signalling cascade. The detailed molecular dissection of mechanisms linking A1R and mGluR1 is outside the scope of this study. Here we discuss some possibilities implied by the existing observations. There are increasing reports (Jordan & Devi, 1999; Bouvier, 2001; Ciruela *et al.* 2001; Ferre *et al.* 2002; Tabata *et al.* 2004; Kubo & Tateyama, 2005) that various GPCRs form hetero-oligomers in which one constituent modulates the other, presumably through direct interaction. Some studies (Ciruela *et al.* 2001; Ferre *et al.* 2002) suggests that A1R can form complexes with and functionally

interact with mGluR1 in the heterologous system. Such direct interaction could occur in Purkinje cells, whereas it is uncertain at present. In an attempt to assess the possibility of complex formation between A1R and mGluR1 in mouse cerebellar neurons, we performed immunoprecipitation assay in the synaptosomal fraction of the cerebellum lysate (Supplemental text and Supplemental Fig. 1). This attempt was hindered because the total amount of A1R protein in the starting material was already low (Supplemental Fig. 1A) and decreased below the threshold of detection after fractionation (Supplemental Fig. 1B). The possibility of complex formation should be re-addressed in future with new tools and techniques. Another possible mechanism is that non-G protein intermediate molecules link A1R and mGluR1. Recently, various transmembrane and cytosolic proteins have been found to interact with and modulate GPCRs (Bockaert *et al.* 2004). The involvement of such a protein remains to be explored in future.

A1R could mediate the $G_{i/o}$ protein-independent depression as well as $G_{i/o}$ protein-dependent augmentation of mGluR1 signalling (Fig. 5). Thus, the net effect of an A1R agonist should be determined as a balance between these opposing actions. The augmentation would obscure the depression when A1R is strongly activated (Fig. 11C). This may explain why CCPA had a weaker depressant effect than R-PIA (Fig. 1B-D). CCPA might induce a higher extent of augmentation than R-PIA, because CCPA is thought to be slightly more potent than R-PIA at the concentrations used (affinities for A1R, 0.4–0.8 nM and 1.17–2 nM, respectively (Poulsen & Quinn, 1998; Klotz, 2000; Fredholm *et al.* 2001)).

The endogenous AR agonist adenosine depressed mGluR1 signalling at both the lowest and highest levels measured in the extracellular fluid in some brain regions (40–400 nM (Ballarin *et al.* 1991); Fig. 10A). This result suggests that the extracellular fluid always contains a high enough level of adenosine to depress mGluR1 signalling. The depression did not display desensitization throughout a prolonged application of adenosine (Fig. 10). Therefore, adenosine may tonically depress mGluR1 signalling to a certain degree under physiological conditions *in situ*. The extent of depression was not much different between 40 nM and 400 nM adenosine (Fig. 10C). One possibility is that A1R is almost fully activated by adenosine at both doses because A1R's dissociation constant for adenosine is 10 nM (Abbracchio *et al.* 1997). Another possibility is that although a larger fraction of the A1R population is activated by adenosine at 400 nM than at 40 nM, depression is compromised at the higher dose as seen with higher doses of R-PIA (Fig. 11C, see above).

When a high dose (400 nM) of adenosine was applied, the amplitude of mGluR1-mediated response recovered only partially after adenosine offset (Fig. 10B). This result indicates that massive A1R activation might result in a

long-term change of mGluR1 signalling under physiological conditions. In addition, the recovery was faster and more complete after the offset of 40 nM adenosine (Fig. 10A) than after the offset of a comparable dose (50 nM) of R-PIA (Fig. 1B). This could be due partly to ecto-/plasma adenosine deaminases that degrade adenosine but not the synthetic agonist (Franco *et al.* 1997; Deussen, 2000).

It was impossible to distinguish A1R–mGluR1 depression from the previously described G protein-dependent inhibition of transmitter release by presynaptic A1R (Dittman & Regeher, 1996) in cerebellar slices (our unpublished data). Our attempt to examine whether A1R antagonists (1,3-dipropyl-8-phenylxanthine (DPPX) and 8-cyclopentyl-1,3-dipropylxanthine) could abolish the depressant effect of endogenous adenosine on glutamate-evoked, mGluR1-mediated currents in the cerebellar slice was also hindered. These antagonist at their effective doses (1–10 μ M for DPPX) turned out to have a side-effect to directly inhibit mGluR1-mediated responses in cultured Purkinje cells (our unpublished data). DPPX at a very low dose (100 nM) had little effect on glutamate-evoked currents in cerebellar slices, whereas its effect at higher doses could not be tested because of the side-effect (our unpublished data). Thus, the *in situ* assessment of the physiological relevance of A1R–mGluR1 depression is difficult at present and awaits the development of new experimental techniques. However, our results together with the previous studies at least suggest a possibility that A1R–mGluR1 depression may influence long-term depression (LTD) of parallel fibre–Purkinje cell (PF–PC) synapses, a form of synaptic plasticity crucial for cerebellar motor learning (Ito, 2002). LTD is induced following repetitive, synchronized transmission at PF–PC and climbing fibre–Purkinje cell (CF–PC) synapses. CFs are reported to release adenosine and/or its precursors in an activity-dependent manner (Do *et al.* 1991). A biochemical study (Balaban *et al.* 1984) shows that activity of 5'-nucleotidase which catalyses the precursors into adenosine increases around the PF–PC synapses following stimulation of CFs. Adenosine depressed the mGluR1-coupled $[Ca^{2+}]_i$ rise (Fig. 13D and E). mGluR1-coupled Ca^{2+} mobilization is a key event for inducing LTD (Ito, 2002). Therefore, CF-derived adenosine may regulate the inducibility of LTD through A1R–mGluR1 depression. Moreover, A1R–mGluR1 depression may also influence information flow carried by slow EPSPs at PF–Purkinje cell synapses, because adenosine reduces an inward current mediated by the channels responsible for slow EPSPs (Fig. 10).

Adenosine is released from various neurons and glia, and thus occurs ubiquitously throughout the brain (see Introduction). Co-localization of A1R and mGluR1 is seen in cortical neurons as well as cerebellar neurons (Ciruela *et al.* 2001). Therefore, A1R–mGluR1 depression may take

place in various brain regions. The findings in this study together demonstrate a novel neuromodulatory action of adenosine in central neurons.

References

- Abbracchio MP, Ceruti S, Brambilla R, Franceschi C, Molorni W, Jacobson KA, von Lubitz DKJE & Cattabeni F (1997). Modulation of apoptosis by adenosine in the central nervous system: a possible role for A₃ receptor. *Ann N Y Acad Sci* **825**, 11–22.
- Aiba A, Kano M, Chen C, Stanton ME, Fox GD, Herrup K, Zwingman TA & Tonegawa S (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* **79**, 377–388.
- Balaban CD, Wurlpel JND & Servers WB (1984). A specific harmaline-evoked increase in cerebellar 5'-nucleotidase activity. *Neurosci Lett* **50**, 111–116.
- Ballarin M, Fredholm BB, Ambrosio S & Mahy N (1991). Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta Physiol Scand* **142**, 97–103.
- Batchlor AM & Garthwaite J (1997). Frequency detection and temporally dispersed synaptic signal association through a metabotropic glutamate receptor pathway. *Nature* **385**, 74–77.
- Bockaert J, Roussignol G, Becamel C, Gavarini S, Joubert L, Dumuis A, Fagni L & Marin P (2004). GPCR-interacting proteins (GIPs): nature and functions. *Biochem Soc Trans* **32**, 851–855.
- Bouvier M (2001). Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* **2**, 274–286.
- Ciruela F, Escriche M, Burgueno J, Angulo E, Casado V, Soloviev MM, Canela EI, Mallol J, Chan W-Y, Lluís C, McIlhinney RAJ & Franco R (2001). Metabotropic glutamate 1alpha and adenosine A₁ receptors assemble into functionally interacting complexes. *J Biol Chem* **276**, 18345–18351.
- Conquet F, Bashir ZI, Davies CH, Daniel H, Ferraguti F, Bordini F, Franz-Bacon K, Reggiani A, Matareses V & Conde F (1994). Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. *Nature* **372**, 237–243.
- Deussen A (2000). Metabolic flux rates of adenosine in the heart. *Naunyn-Schmiedeberg's Arch Pharmacol* **362**, 351–363.
- Dittman JS & Regeher WG (1996). Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. *J Neurosci* **16**, 1623–1633.
- Do KQ, Vollenweider FX, Zollinger M & Cuenod M (1991). Effect of climbing fibre deprivation on the K⁺-evoked release of endogenous adenosine from rat cerebellar slices. *Eur J Neurosci* **3**, 201–208.
- Dunwiddie TV & Masino SA (2001). The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* **24**, 31–55.
- Ferre S, Karcz-Kubicha M, Hope B, Popoli P, Burgueno J, Gutierrez MA, Casado V, Fuxe K, Goldberg SR, Lluís C, Franco R & Ciruela F (2002). Synergistic interaction between adenosine A_{2A} and glutamate mGlu5 receptors: implication for striatal neuronal function. *Proc Natl Acad Sci U S A* **99**, 11940–11945.

- Finch EA & Augustine GJ (1998). Local calcium signalling by inositol-1,4,5-triphosphate in Purkinje cell dendrites. *Nature* **396**, 753–756.
- Franco R, Casado V, Ciruela F, Saura C, Mallol J, Canela EI & Lluis C (1997). Cell surface adenosine deaminase: much more than an ectoenzyme. *Prog Neurobiol* **52**, 283–294.
- Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G & Wasserman W (2000). Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg Arch Pharmacol* **362**, 364–374.
- Fredholm BB, Ijzerman AP, Jacobson KA, Klotz K-N & Linden J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**, 527–552.
- Haas HL & Selbach O (2000). Functions of neuronal adenosine receptors. *Naunyn-Schmiedeberg Arch Pharmacol* **362**, 375–381.
- Hartmann J, Blum R, Kovalchuk Y, Adelsberger H, Kuner R, Durand GM, Miyata M, Kano M, Offermanns S & Konnerth A (2004). Distinct roles of Galpha(q) and Galpha11 for Purkinje cell signaling and motor behavior. *J Neurosci* **24**, 5119–5130.
- Hirono M, Yoshioka T & Konishi S (2001). GABA_B receptor activation enhances mGluR-mediated responses at cerebellar excitatory synapses. *Nature Neurosci* **4**, 1207–1216.
- Horn R & Marty A (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* **92**, 145–159.
- Houamed KM, Kuijper JL, Gilbert TL, Haldeman BA, O'Hara PJ, Mulvihill ER, Almers W & Hagen FS (1991). Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. *Science* **252**, 1318–1321.
- Ichise T, Kano M, Hashimoto K, Yanagihara D, Nakao K, Shigemoto R, Katsuki M & Aiba A (2000). mGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse elimination, and motor coordination. *Science* **288**, 1832–1835.
- Ito M (2002). The molecular organization of cerebellar long-term depression. *Nat Rev Neurosci* **3**, 896–902.
- Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Gimenez-Llort L, Escorihuela RM, Fernandez-Teruel A, Wiesenfeld-Hallin Z, Xu X-J, Hardemark A, Betsholtz C, Herlenius E & Fredholm BB (2001). Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A₁ receptor. *Proc Natl Acad Sci U S A* **98**, 9407–9412.
- Jordan BA & Devi LA (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* **399**, 697–700.
- Kano M, Hashimoto K, Kurihara H, Watanabe M, Inoue Y, Aiba A & Tonegawa S (1997). Persistent multiple climbing fiber innervation of cerebellar Purkinje cells in mice lacking mGluR1. *Neuron* **18**, 71–79.
- Kim JK, Kin YS, Yuan JP, Petralia RS, Worley PF & Linden DJ (2003). Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1. *Nature* **426**, 285–291.
- Klinger M, Freissmuth M & Nanoff C (2002). Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signal* **14**, 99–108.
- Klotz K-N (2000). Adenosine receptors and their ligands. *Naunyn-Schmiedeberg Arch Pharmacol* **362**, 382–391.
- Kubo Y & Tateyama M (2005). Towards a view of functioning dimeric metabotropic receptors. *Curr Opin Neurobiol* **15**, 289–295.
- Lliano I, Dressen J, Kano M & Konnerth A (1991). Intradendritic release of calcium induced by glutamate in cerebellar Purkinje cells. *Neuron* **7**, 577–583.
- Maejima T, Hashimoto K, Yoshida T, Aiba A & Kano M (2001). Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. *Neuron* **31**, 463–475.
- Maejima T, Oka S, Hashimoto Y, Ohno-Shosaku T, Aiba A, Wu D, Waku K, Takayuki Sugiura T & Kano M (2005). Synaptically driven endocannabinoid release requires Ca²⁺-assisted metabotropic glutamate receptor subtype 1 to phospholipase C E signaling cascade in the cerebellum. *J Neurosci* **25**, 6826–6835.
- Marty A & Neher E (1995). Tight-seal whole-cell recording. In *Single-Channel Recording*, 2nd. edn, ed. Sakmann B & Neher E, pp. 31–52. Plenum, New York.
- Masu M, Tanabe Y, Tsuchida K, Shigemoto R & Nakanishi S (1991). Sequence and expression of a metabotropic glutamate receptor. *Nature* **349**, 760–765.
- Miyashita T & Kubo Y (2000). Extracellular Ca²⁺ sensitivity of mGluR1alpha induces an increase in the basal cAMP level by direct coupling with Gs protein in transfected CHO cells. *Receptors Channels* **7**, 77–91.
- Miyata M, Finch EA, Khiroug L, Hashimoto K, Hayasaka S, Oda SI, Inouye M, Takagishi Y, Augustine GJ & Kano M (2000). Local calcium release in dendritic spines required for long-term synaptic depression. *Neuron* **28**, 233–244.
- Nyce JW (1999). Insight into adenosine receptor function using antisense and gene-knockout approaches. *Trends Pharmacol Sci* **20**, 79–83.
- Poulsen S-A & Quinn RJ (1998). Adenosine receptors: new opportunities for future drugs. *Bioorganic Med Chem* **6**, 619–641.
- Reppert SM, Weaver DR, Stehle JH & Rivkees SA (1991). Molecular cloning and characterization of a rat A1 adenosine receptor that is widely expressed in brain and spinal cord. *Mol Endocrinol* **5**, 1037–1048.
- Ribeiro JA, Sebastiao AM & De Mendoca A (2003). Adenosine receptors in the nervous system: pathophysiological implications. *Prog Neurobiol* **68**, 377–392.
- Rudolph KA, Schubert P, Parkinson FE & Fredholm BB (1992). Neuroprotective role of adenosine in cerebral ischaemia. *Trends Pharmacol Sci* **13**, 439–445.
- Saitow F & Konishi S (2000). Excitability increase induced by beta-adrenergic receptor-mediated activation of hyperpolarization-activated cation channels in rat cerebellar basket cells. *J Neurophysiol* **84**, 2026–2034.
- Sato M, Tabata T, Hashimoto K, Nakamura K, Nakao K, Katsuki M, Kitano J, Moriyoshi K, Kano M & Nakanishi S (2004). Altered agonist sensitivity and desensitization of neuronal mGluR1 responses in knock-in mice by a single amino acid substitution at the PKC phosphorylation site. *Eur J Neurosci* **20**, 947–955.

- Schubert P, Ogata T, Marchini C, Ferroni S & Rudolph K (1997). Protective mechanisms of adenosine in neurons and glial cells. *Ann N Y Acad Sci* **825**, 1–10.
- Selbie LA & Hill SJ (1998). G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signaling pathways. *Trends Pharmacol Sci* **19**, 87–93.
- Shigemoto R, Abe T, Nomura S, Nakanishi S & Hirano T (1994). Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. *Neuron* **12**, 1245–1255.
- Svenningsson P, Le Moine C, Kull B, Sunahara R, Bloch B & Fredholm BB (1997). Cellular distribution of adenosine A_{2A} receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. *Neuroscience* **80**, 1171–1185.
- Tabata T, Aiba A & Kano M (2002). Extracellular calcium controls the dynamic range of neuronal metabotropic glutamate receptor responses. *Mol Cell Neurosci* **20**, 56–68.
- Tabata T, Araishi K, Hashimoto K, Hashimoto-dani Y, Van der Putten H, Bettler B & Kano M (2004). Ca²⁺ activity at GABAB receptor constitutively promotes metabotropic glutamate signaling in the absence of GABA. *Proc Natl Acad Sci U S A* **101**, 16952–16957.
- Tabata T, Haruki S, Nakayama H & Kano M (2005). GABAergic activation of an inwardly rectifying K⁺ current in mouse cerebellar Purkinje cells. *J Physiol* **563**, 443–457.
- Tabata T, Sawada S, Araki K, Bono Y, Furuya S & Kano M (2000). A reliable method for culture of dissociated mouse cerebellar cells enriched for Purkinje neurons. *J Neurosci Meth* **104**, 45–53.
- Takechi H, Eilers J & Konnerth A (1998). A new class of synaptic response involving calcium release in dendritic spines. *Nature* **396**, 757–760.
- Tateyama M & Kubo Y (2006). Dual signaling is differentially activated by different active state of the metabotropic glutamate receptor 1α. *Proc Natl Acad Sci U S A* **103**, 1124–1128.
- Tempia F, Alojado ME, Strata P & Knöpfel T (2001). Characterization of the mGluR1-mediated electrical and calcium signaling in Purkinje cells of mouse cerebellar slices. *J Neurophysiol* **86**, 1389–1397.
- Wirkner K, Assmann H, Koles L, Gerevich Z, Franke H, Norenberg W, Boehm R & Illes P (2000). Inhibition by adenosine A_{2A} receptors of NMDA but not AMPA currents in rat neostriatal neurons. *Br J Pharmacol* **130**, 259–269.

Acknowledgements

We thank Dr T. Shirao and Dr T. Hirata for gift of experimental materials, S. Haruki for cell culture, and N. Inoike for technical assistance. This work was supported by the Grants-in-Aid for Scientific Research (18019022 and 17700305 to T.T., 16680014 to K.H., 17023021 and 17100004 to M.K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Swedish Science Research Council to B.B.F. (grant no. 2553).

Author's present address

Y. Sekino: Division of Neuronal Network, Department of Basic Medical Sciences, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan.

Supplemental material

Online supplemental material for this paper can be accessed at: <http://jp.physoc.org/cgi/content/full/jphysiol.2007.129866/DC1> and <http://www.blackwell-synergy.com/doi/suppl/10.1113/jphysiol.2007.129866>